PROBABLE NOVEL PROBIOTICS: EPS PRODUCTION, CHOLESTEROL REMOVAL AND GLYCOCHOLATE DECONJUGATION OF LACTOBACILLUS PLANTARUM GA06 AND GA11 ISOLATED FROM LOCAL HANDMADE-CHEESE

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ABSTRACT

In this study, it was aimed to investigate the effect of exopolysaccharide produced by 2 novel probiotic strains (Lactobacillus plantarum GA06 and GA11), isolated from local handmade cheese, on cholesterol mechanism and bile deconjugation. These two strains were thought to play an important role in cholesterol removal and may contribute to intestinal microbiota positively. The bacterial 16S rRNA gene sequencing analysis was used for molecular identification of the strains. Exopolysaccharide production of the strains was identified by the phenol-sulphuric acid method whereas cholesterol removal and glycocholate deconjugation activity were evaluated via spectrophotometric methods. The exopolysaccharide production of L. plantarum GA11 and GA06 was determined as 96.5 and 32.5 mg/L, and the cholesterol removal amounts were found as 36.7% and 28.6% respectively. Glycocholate deconjugation activity of GA11 was detected higher than of GA06. A statistically significant difference was observed between the EPS production capacity of the strains and the removal of cholesterol (p<0.05). Also, a statistically significant correlation was found both between the EPS production capacity- cholesterol removal and between EPS production capacity-glycocholate deconjugation. According to the results of our study, L. plantarum GA11 strain appears to be a good and novel probiotic strain that has high probiotic potential. Further studies can be confirmed to determine specific more crucial health benefits.

Keywords: Novel probiotics, L. plantarum, EPS production, Cholesterol removal, Glycocholate deconjugation

INTRODUCTION

Trillions of microorganisms play a major role in human health and disease. In particular, intestines are seen as “a basic living area” for many of these microorganisms. There are numerous studies indicating that intestinal microbiota is important for human beings. Most of the chronic diseases are emphasized the importance of human microbiota. Probiotics have protective effects on the development of lactose intolerance, cholesterol-lowering, immune system supportive, antibacterial, intestinal infections prevention, intestinal flora and digestive mechanism (Ley et al., 2006; Ursel et al., 2014; Kumar and Chordia, 2017; Wang et al., 2017).

For the production of probiotic microorganisms industrially or naturally present in foods, there are some criteria defined by LABIP (Lactic Acid Bacteria Industrial Platform) in order to promote the survival of the probiotics in the digestive system (Ewaschuk and Dieleman, 2006). Firstly, probiotic microorganisms; should be of human origin, resistance to digestive enzymes and bile salts, and have antimicrobial activity (Ooi and Liong, 2010). Apart from those, one of the desirable characteristics for an organism to be selected as a probiotic is the ability of bile salts deconjugation. This is an important issue for individuals associated with coronary heart disease having high cholesterol levels in the serum (Lim et al., 2004). Recent studies have reported that blood cholesterol levels have remarkably decreased in individuals who are interested in dieting with probiotic foods including L. plantarum, B. bifidum and L. bulgaricus (Roos and Katan, 2000; Ooi and Liong, 2010; Zanotti et al., 2015).

Two main hypotheses for cholesterol removal mechanisms of probiotic microorganisms have been proposed which are bile salts deconjugation via the bile salt hydrolase (BSH) enzyme and cholesterol assimilation in the cell membrane (Noriega et al., 2006; Jarrocki et al., 2014; Sledlackova et al., 2015). Deconjugation of bile acids is thought to help reducing serum cholesterol levels via decreasing the amount of bile acid that is returned to the liver by the enterohepatic cycle so that increasing the production of bile acids via using cholesterol as substrate. This hydrolysis taking place during the enterohepatic circulation is catalyzed by the BSH enzyme produced through intestinal microbiota such as Lactobacillus, Bifidobacterium, Enterococcus, Bacillus, Clostridium, Faobacterium, Bacteroides, Pseudomonas and Peptostreptococcus (Floh, 2002; Ooi and Liong, 2010).

Cholesterol assimilation by probiotic microorganism is the second main mechanism thereby reducing lipids and cholesterol levels. It was reported that cholesterol reduction in media was caused by the acidic environment as a result of bile salts decomposition, cholesterol is precipitated with free bile acids, not metabolized by the microorganisms (Klaver et al., 1993; Shehata et al., 2016). Further studies are required to elucidate the exact mechanism(s) of the molecular basis on cholesterol lowering especially on humans. Sodium glycocholate is the predominant component of bile salts in the human intestine, therefore, the strains that deconjugate sodium glycocholate may be more effective in lowering serum cholesterol (Choi et al., 2015; Mahmoudi et al., 2017).

It was observed in the previous studies that there is a correlation between the EPS production of the probiotic strains and the cholesterol removing mechanism. It was suggested that these polysaccharides may have a similar effect to dietary fiber foods if the amount of EPS produced by the cells is high and thus the cholesterol can be increased by binding to the EPS produced by the bacteria to increase the amount of cholesterol excreted by the bacteria, thus reducing the amount of cholesterol that is absorbed from the intestine into the blood (Ooi and Liong, 2010; Ayyash et al., 2018; Bhat and Bajaj, 2018).

In this study, it was aimed to investigate the effect of exopolysaccharide, produced by Lactobacillus plantarum GA06 and GA11 strains, on cholesterol mechanism and bile deconjugation. These two strains were thought to play an important role in cholesterol removal and may contribute to the intestinal microbiota positively.
**MATERIALS AND METHODS**

**Isolation and culture conditions of Lactobacillus spp.**

Lactobacillus spp. were obtained by classical cultivation methods from local handmade-cheese. De Man Rogosa and Sharpe (MRS, Merck-Germany) media were used in the growth and isolation of bacteria. After incubation at 37°C for 18-24 h, the probable Lactobacillus spp. colonies were detected by Gram reaction and colony morphology. All strains were activated two times before the usage for all tests.

**Biochemical and molecular identification of Lactobacillus spp.**

All of the strains were characterized based on the fermentation of carbohydrates using API 50CHL kit (Biomerieux, France). According to color change, the results were evaluated as positive or negative. The results were identified by using the database (V5.1) and apw™ identification software.

For the molecular identification of microorganisms, 16S rRNA analysis was used. Firstly, DNA was extracted by Invitrogen Genomic DNA Extraction Kits (Thermo Fisher Scientific) from overnight cultures of pure LAB strains in 5 ml MRS broth and centrifuged at 12000 × g for 2 min at 4 °C. 16S rRNA gene was amplified by polymerase chain reaction (PCR) to identify isolated bacteria by molecular methods. The universal primers were used 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) for 16S rDNA. The PCR protocol: 94°C 90 s, 48°C 35 s, 50°C 35 s, 72°C 10 s, 33 cycles and a final extension step at 72°C for 3 min, 4°C for 24 h. Sequencing was made via Applied Biosystems BigDye terminator cycle sequencing version 2.0 kit (Applied BioSystems, Foster City, Calif.). Products were purified with Sephadex spin columns (Cold Spring Harbor Protocol, 2002, California), resolved with DNA sequencing system (Applied Biosystems, 3130XL).

**EPS production capacity**

EPS extraction was performed according to Valérie et al., (1999)’s method. Activated isolates of Lactobacillus spp. were firstly adjusted to OD600 nm = 0.600 and inoculated 2% in MRS medium and incubated at 42°C in anaerobic conditions for 24 hours. After 24 hours, 1 mL of culture was boiled at 96°C for 10-15 minutes and then allowed to cool to room temperature. 17% of the sample on 1 mL sample was centrifuged for 13 min at 13 000 rpm with 85% TCA. The supernatants were mixed with an equal volume of ethanol, then centrifuged again. Samples were dissolved by adding ethanol to the pellets for the second time. The dissolved samples were again centrifuged to precipitate. Precipitated samples were dissolved in 1 mL dH2O and the amount of EPS was determined via penol-sulphuric acid method determined by Dubois et al., (Dubois et al., 1956).

Glucose was used for the standardization of EPS studies (Fig. 1).

![Figure 1 L. plantarum GA06 (A) and GA11 (B) microscopy.](image)

**Cholesterol removal**

A previously modified method by Alp Avci (2016) was used for the removal. Cholesterol solution, freshly prepared with ethyl alcohol, was added to MRS broth up to 100 µg/ml, final concentrations. These solutions were inoculated as 2% into the media for each strain and incubated at 37 °C for 16-18 h. Bacterial cells were separated via centrifugation at 10,000 g for 20 min, cholesterol in the supernatant was spectrophotometrically measured at 600 nm. Then they were dried at 80 °C and dissolved in distilled water. Subsequently, the cholesterol assay as described by Gilliland et al., (1985) was performed. The percentage of assimilations was calculated by the equation established as below:

\[
\text{Cholesterol assimilation (%) =} \frac{\text{cholesterol amount in inoculated medium}}{\text{cholesterol amount in control medium}} \times 100
\]

**Deconjugation of sodium glycocholate**

The MRS mediums were supplemented with 2 mg/ml sodium glycocholate. Strains were inoculated by 1% (except control groups), incubated for 18-20 h at same temperature. Based on measuring free cholic acid amount unleashed through each bacterium, deconjugation ability was detected as spectrophotometrically (Abs:660nm, BiochromeLibra, UK) (Alp Avci, 2016).

**Statistical analysis**

Statistical analysis was applied using SPSS 22.0 software (SPSS Inc., USA). Data are reported as mean±SD. The significance level was set at 0.05. Normality distribution was assessed by Shapiro-Wilk test. The non-parametric Kruskal Wallis H-test was applied for comparison of differences between means. In order to find the association between the parameters that were studied, Spearman correlation coefficient was used.

**RESULTS AND DISCUSSION**

Certain strains of LAB are able to synthesize exopolysaccharides which play a crucial role in the food industry as emulsifying, gelling and stabilizing, moisture-retaining and thickening agents (Duboc and Mollet, 2001; Jolly et al., 2002; Ahmad et al., 2015, Venugopal, 2019). EPS has been claimed to have an anti-tumor effect on human health, immune activating and cholesterol-lowering effect. Therefore, EPS has great potential for its application even in the pharmaceutical industry (Chun-lei et al., 2014). Moreover, Tok and Aslim (2010) suggested that bacterial EPS interacts with the cholesterol in the environment and binds it like dietary fiber.

In this study, the effect of exopolysaccharide that produced by L. plantarum GA06 and GA11 strains on cholesterol mechanism was investigated. The strains were thought to play a major role in cholesterol removal and contribute intestinal microbiota positively. In our study, the amount of EPS production through L. plantarum GA06 and GA11 was found 32.50 and 96.45 mg/L, respectively (Fig.3). When similar studies in the world are examined, it is seen that EPS production is important for probiotic microorganisms. L. plantarum (HQC259238) isolated from Traditional-Chinese fermented foods had high EPS production capability (859 mg/L) (Feng et al., 2012). EPS amounts of L. plantarum YO175 and OF101, which were obtained from Nigerian traditional fermented cereal gruel ‘ogi’ and grown on sucrose modified mMRS, were found 1.36 g/L and 2.18 g/L, respectively (Adesulun-Dahunsi et al., 2018). Khalil et al., (2018) found that EPS production, via L. plantarum TEMP strain obtained from traditional Malaysian fermented foods, was 80 mg/L, EPS yield of L. fermentum PIC7 was 750 mg/L. Van Der Meulen et al., (2007) reported that EPS amounts of Lactobacillus strains isolated from dairy and cereal products, were ranged from 0.8 and 1.7 g/L. EPS levels of L. plantarum strains were determined via spectrophotometrically at 660 nm for each strain and incubated at 37 °C for 16-18 h as below; the EPS produced was 250 mg/L by Imran et al., (2016). Ismail and Namboothiri (2010) found that the L. plantarum MTCC 9510, produce 210 mg/L EPS in media (added 2% sucrose). In previous work, yield of EPS from L. plantarum MCC2034 showed differences in MRS broth with glucose, mod-MRS broth with sucrose and synthetic medium with lactose producing 251 mg/L, 1.059 mg/L, and 1.060 mg/L, respectively. Therefore, it could be suggested that EPS production levels can depend on carbon source, sugar carbohydrate types in the culture medium, and bacterial strain (Badel et al., 2011). Seeo et al. (2015) isolated L. plantarum YML009 on MRS supplemented with 10% glucose, producing 260 mg/L EPS. The media components have affected EPS production because of especially carbohydrate/carbon sources in the medium.

![EPS standard](image)

**Figure 2 The standard EPS graphic that prepared with glucose.**
Cholesterol is a necessary lipid derivative for all living things. This substance is a major component of the cell membrane, bile acids, steroid hormones and the precursor molecule of vitamin D and has an important role in metabolic activities. Until now, some studies have shown that probiotics like L. acidophilus, B. bifidum, and L. bulgaricus significantly reduce cholesterol levels in coronary heart disease (Ooi and Liang, 2010). Lowering blood cholesterol levels have been increasing because of probiotics usage. Nqogang et al., (2016) observed two of three isolates from palm wine were L plantarum which were able to decrease cholesterol level in MRS broth (p<0.05). L. plantarum 2R30 strain assimilated 51.25% cholesterol in the media while L. plantarum 2R36 had 55.22% cholesterol reduction. L. plantarum and L. paracasei isolated from cheese were investigated in-vitro to decrease cholesterol (Belvoso et al., 2009). L. plantarum and L. paracasei showed 19.4 % and 6.8 % cholesterol removal, respectively. Guo et al. (2011) found 23.55- 54.08 % cholesterol removal ability of 6 L. plantarum strains obtained from home-made fermented cream in Mongolia, China. In our study, the cholesterol removal ability of L. plantarum GA06 was 28.56 % while L. plantarum GA11 has 36.57 % (Fig. 3). A significant difference was observed between EPS production and cholesterol removal (p<0.05). Also and, a statistically positive correlation was determined between EPS production and cholesterol removal (Fig. 4). Most of the studies reported that there is a correlation between the EPS production of the probiotic strains and the cholesterol removing mechanism, so our research showed parallelism to this situation. Compared with the studies, L. plantarum has an average EPS production which might result in average cholesterol removal.

Anandharaj and Sivasankari (2014) investigated the deconjugation ability of isolated L. oris strains from mother’s milk. HM168 strain had the highest deconjugation ability for taurocholate (0.98 mM) and glycocholate (0.89 mM). In another research studied with B. pseudocatenulatum G4, which is thought as a probable probiotic strain, was isolated from infant feces samples. It showed to deconjugation of all bile salts at the level of 0.25 mM at pH 5.7 (Rasti et al., 2011). In our study, the glycocholate deconjugation capacity of L. plantarum GA06 and GA11 strains were 1.02±0.1 mM (19.42%) and 1.30±0.2 mM (31.84%), respectively (Fig. 3). Compared with the previous studies, L. plantarum GA11 has a dramatically high concentration of glycocholate deconjugation. Since the strains which deconjugate sodium glycocholate may be more effective in lowering serum cholesterol (Mahmoudi et al., 2017), L. plantarum GA11 could have more hypocholesteremic effect in order to prevent hypercholesterolemia. A statistically significant difference was found between the EPS production and glycocholate deconjugation (p<0.05). Moreover, a statistically significant correlation was found between EPS production and deconjugation.

CONCLUSIONS

Today, many probiotic microorganisms are discovered, however, it is important that these microorganisms are probiotic microorganisms with superior characteristics. In our study, cholesterol removal and glycocholate deconjugation of the strains were evaluated together in order to reveal the superior probiotic microorganisms. L. plantarum GA11 strain showed important results in the EPS production level, cholesterol removal and high concentration of glycocholate deconjugation in this study. According to the results of our study, L. plantarum GA11 strain appears to be a good and novel probiotic strain that has high probiotic potential. Further studies can be confirmed to determine more crucial health benefits.

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